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Original Article

Loss of Mac-1 causes lung respiratory failure through affecting type I alveolar epithelial cells

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Abstract: Type I alveolar cell damage associates with a variety of lung diseases, and severe damages can lead to respiratory failure. Mac-1 as a member of integrin family has been studied for a long time. This study investigated the relationship between Mac-1 deficiency and respiratory failure in Mac-1 Knockout (Mac-$1^{-/-}$) mice. C_{57} mice were used as a control. The newborn survival rate of Mac-$1^{-/-}$ mice was calculated; HE staining of mice lung tissue was performed for histological tests; Western Blotting, Q-PCR detection were used to detect the expression of type I and type II alveolar epithelial cells, as well as alveolar surfactant secreted by type II alveolar epithelial cells. Birth survival rate of Mac-$1^{-/-}$ mice was significantly lower than that of C_{57} mice; in lung floating experiment, the lung of C_{57} mice were floating upwards, but for Mac-$1^{-/-}$ mice, the lungs sank downwards to the bottom of the EP tube. Compared with C_{57} mice, the ProSP-C, as the specific protein of type II alveolar epithelial cells, and the alveolar surfactants in Mac-$1^{-/-}$ mice had no significant differences, and the structure and function were basically complete. However, western blotting showed that expression of T1α, Aqp5 and Snx5 in Mac-$1^{-/-}$ mice, as the specific proteins of type I alveolar epithelial cells, was decreased significantly than those in C_{57} mice ($P<0.05$). Mac-1 may play an important role in the development of respiratory failure. Lack of Mac-1 leading to respiratory failure is not affected by type II alveolar epithelial cells or their secretive surfactant, but rather by reducing type I alveolar cells.

Keywords: Mac-1, type I alveolar epithelial cells, respiratory failure, type II alveolar epithelial cells

Introduction

Respiratory failure is a common disease in clinic, and severe cases, if not get the timely and effective treatments, can lead to a series of serious complications, such as multiple organ dysfunction syndrome (MODS), and eventually death [1, 2]. Alveolar epithelial cells are composed of type I and type II alveolar cells, of which, type II alveolar epithelial cells are synthesized by the original stem cells, having the potential to differentiate into type I alveolar epithelial cells, which can synthesize and secrete alveolar surfactant, and are of great significance to maintain the stability of alveolar [3, 4]. There are many in-depth studies on the type II alveolar cells in the pathogenesis of lung injury. Type I alveolar epithelial cell, a large and flat cell, covers more than 95% of the alveolar surface area, which is the target cell of various types of damage. Type I alveolar epithelial cells may have a variety of biological functions, besides being involved in the formation of air-blood barriers, and they can also transport water and ions with certain immune regulating functions [5, 6]. However, the role of type I alveolar cells in the pathogenesis of lung injury remain unclear.

Mac-1 is a member of β2 integrin family. As an important adhesion molecule, it is involved in the body defense and immune response, and expressed widely in partial white blood cells, such as neutrophils, monocytes, eosinophilic cells and NK cells, with few expressions in T cells, macrophages, and B cells [7-9]. Lack of Mac-1 affects the migration and tissue infiltration of white blood cells, which play a biological function in the inflammatory response, and inflammation has a close relationship with the occurrence and development of lung injury [10, 11]. However, it is still unclear about the role of

Mac-1 in the occurrence and development of lung injury, especially of its role in the type I alveolar epithelial cells. This article focuses on the role of Mac-1 in respiratory failure and its mechanism.

Materials and methods

Materials

Experimental animals and breeding: Mac-1 Knockout mice (B6.129S6-Itgam tm1Myd/J), homozygous (Mac-$1^{-/-}$), were purchased from the Jackson Laboratory (US), coded as 003991. C_{57} BL/6J mice (hereinafter referred to as C_{57}) were used as the background control mice, purchased from in the Medical Laboratory Animal Center of Shandong Province, the production license was SCXK 2008-0002. These mice were raised in VMC64S7 independent delivery and return air purification baskets (Suhang Experimental Animals Equipment Factory) in SPF environment, room temperature was 22 to 28°C, relative humidity was 50% to 70%, automatic light control (12 h light/12 h dark). Feed were purchased from Shandong Animal Center, which underwent 60Co irradiation sterilization. Drinking water was sterilized urban water.

Mice were used for all experiments, and all procedures were approved by the Animal Ethics Committee of People's Hospital of Laizhou City.

Main instruments and reagents

PCR (Biometra Company); Stereoscopic microscope (Motic Company); Proteinase K, dNTP (Shanghai Univ-bio Company); PCR primers (Invitrogen Trading (Shanghai) Co., Ltd); Taq polymerase, Tris saturated phenol, chloroform (sigma Co., Ltd).

Methods

Mac-$1^{-/-}$ gene identification: Identification sequence of Mac-1 knockout mice: mutations stripe: primer 1: 5'-TAG GCT ATC CAG AGG TAG AC-3'; Primer 2: 5'-ATC GCC TTC TTG ACG AGT TCA-3', amplification bands were 700 bp. Wild type stripe: primer 1: 5'-TAG GCT ATC CAG AGG TAG AC-3'; Primer 2: 5'-CAT ACC TGT GAC CAG AAG AGC-3', amplification bands were 325 bp. The PCR reaction conditions: denaturations were at 94°C for 3 minutes, 94°C for 30 seconds, 58°C for 1 minute, 72°C for 2 minutes, with 35 cycles; Extensions were at 72°C for 2 minutes, annealing was at 10°C.

Study group and the control

The male and female C_{57} mice, in the proportion of 1:2, mated in the full siblings mating way, and the pregnant female mice, as the control group, were placed separately in a cage. The male and female Mac-$1^{-/-}$ mice, in the proportion of 1:2, mated in the full siblings mating way, and the pregnant female mice, as the experimental group, were placed separately in another cage. The feeding conditions for the two mice were the same. All operations were conducted within the scope of ethics.

Lung floating experiment

The lung tissues, after Mac-$1^{-/-}$ mice were sacrificed, were immediately removed to a centrifuge tube with 0.5 ml of PBS.

HE staining

The lung tissues were placed in formalin to be fixed for one night, and then dehydrated. Dehydration was performed as follows: 70% ethanol for 3 h, 80% ethane for 3 h, 95% ethanol for 2 h, 100% ethanol I for 1.5 h, 100% ethanol II for 1.5 h, Xylene I/II each for 0.5 h, paraffin I for 1 h, paraffin II for 2 h (60°C); Conventional slice and the thickness was 3 μm. Dewaxing: per the conventional dewaxing method: 10 minutes were taken in xylene, anhydrous ethanol, 95% ethanol, 90% ethanol, 85% ethanol and 80% ethanol respectively. Conduct hematoxylin stain for 1 min, and wash it with water to blue, then conduct eosin stain for 10 sec, wash it with water, dry it and mount followed by observation under the microscope, take 20× of the middle part in the visual field, and then three different pathology technicians read and diagnosed it.

Immunofluorescence staining

After the tissue was fixed, use sucrose for gradient dehydration, freeze and then slice by 6 mm of thickness, repair at high temperature for 5 min. After it cools, PBS washed it for three times, five minutes per time followed by addition of 10% BSA for 50 min, and then conduct primary antibody staining overnight, rewarm on the second day for 40 min, wash it with PBS

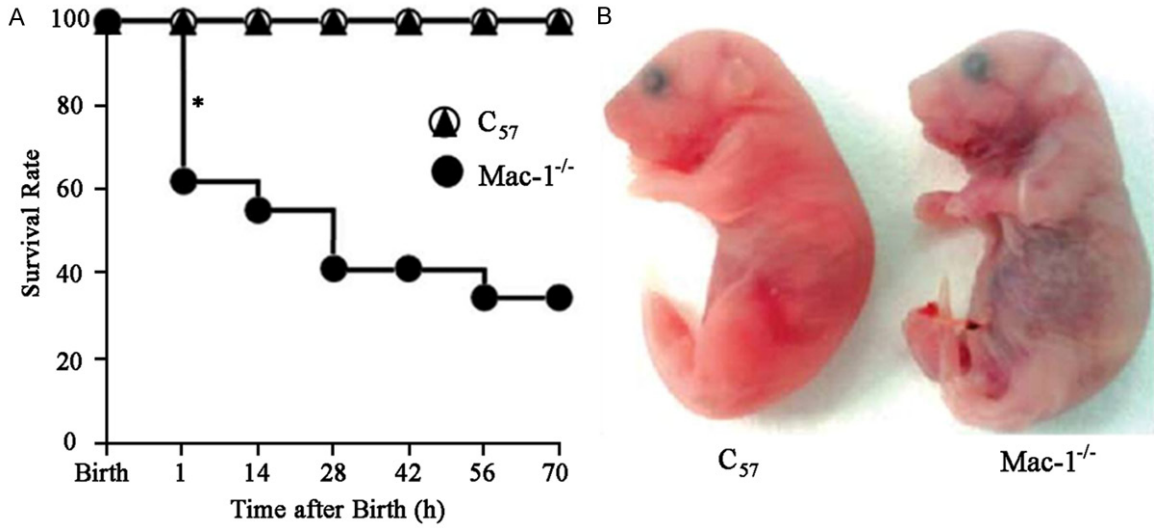

Figure 1. A. The Survival Rates of C_{57} and Mac-$1^{-/-}$ Newborn Mice, *indicates P<0.05; B. The Obvious Cyanosis of Mac-$1^{-/-}$ Newborn Mice Body, Compared with C_{57} Mice.

three times, 5 min for each time, then conduct second antibody fluorescence staining. Primary antibody was proSP-C (anti-rabbit), purchased from Santa, 1:100; second antibody was anti-rabbit (488 nm), purchased from Santa, 1:100. Dry mounting. Calculate the ratio of positive cell number to the area of vision field by J image software. Compare the difference of positive rates that was the positive expression difference. Get rid of bubble from the plate before sampling.

Western blotting

Tissue proteins were extracted from protein kit, and use BCA for quantitative analysis. Prepare BCA solution by the ratio of A:B to 50:1. Take 2 μl of collected cell supernatant, add 18 μl of PBS and 200 μl of AB mixed solution. All protein samples were adjusted to the same concentration, and add 1× bromphenol blue, which accounted for 1/5 of the total volume. All protein samples were transferred to the same concentration, and then sample, while adding 6 μl of protein marker. run in concentration gel at the initial voltage of 80 V, and then raise to 120 V to run in the separation gel. finish the process when the target protein swam to 1 cm above the edge of the gel. immerse PVDF membrane in methyl for 5 min. Constant current was at 250 mA for 90 minutes. Take the membrane out of electronic transfection pool; rinse slightly with TBST, swing slowly in 5% skim milk sealing solution for 1 h. Rinse slightly with TBST. Incubate with primary antibody overnight, and warm it at room temperature for 40 minutes on the second day. Rinse the membrane with TBST three times, 5 minutes for each time. Choose the secondary antibody per the primary antibody, and swing slightly at room temperature for 1 hour. After incubation with the secondary antibody, wash the membrane with TBST three times, 5 minutes for each time. The washed PVDF film was developed with ECL with the configuration of ECL liquid being A:B as 1:1.

Statistical analysis

Western-Blot results were processed by the Image-J software which were repeated at least three times and showed as mean ± standard deviation (SD). SPSS11.0 was used for statistical analysis. Comparison of difference was assessed by student t test. P<0.05 indicated the difference was statistically significant.

Results

The survival rates of C_{57} and Mac-$1^{-/-}$ newborn mice

The survival rate of C_{57} newborn mice was 100% at 10 hours after birth (**Figure 1A**), but the survival rate of Mac-$1^{-/-}$ newborn mice reduced to 60% at 1 hour after birth, which was decreased more with time increasing, and at 70 hours after birth, it was reduced to less than

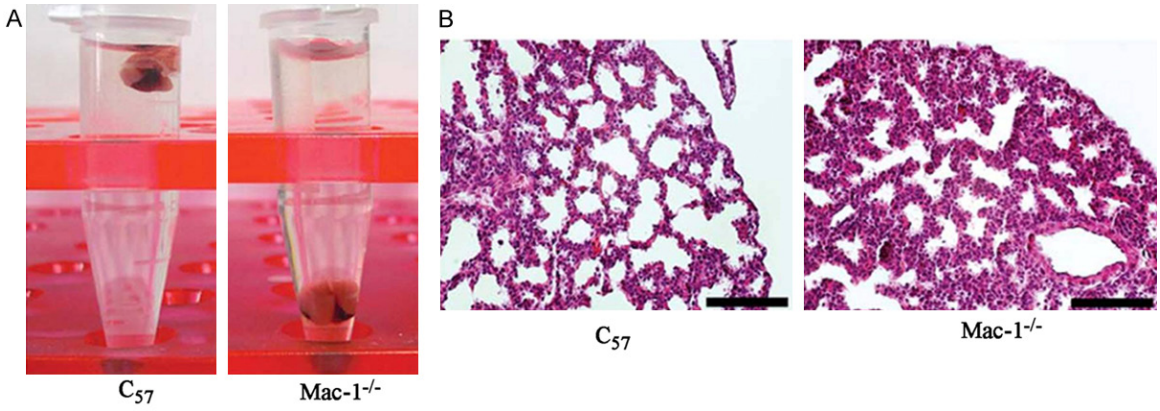

Figure 2. Alveolar Layer Thickening of Mac-1$^{-/-}$ Mice. A. Indicated that compared with C_{57} mice, Mac-1$^{-/-}$ mice may have respiratory failure at birth. B. Showed that compared with that of C_{57} mice, the alveolar layer of mac-1$^{-/-}$ mice became thickening.

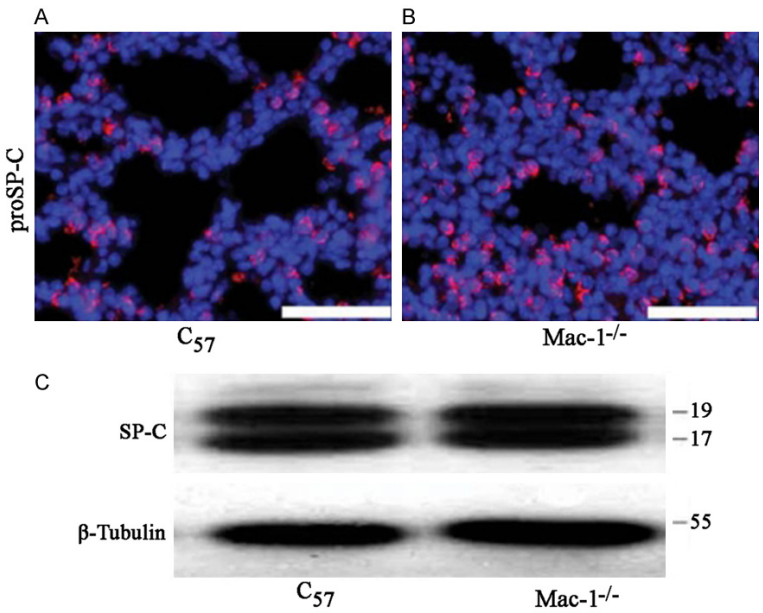

Figure 3. The difference between the alveolar surfactant proteins of type II alveolar epithelial cells in C_{57} and Mac-1$^{-/-}$ mice was not significant. A and B. Showed the results of proSP-C detected by immunofluorescence assay. C. Showed the western blotting results of SP-C.

40%. The difference of survival rates between these two groups was statistically significant ($P<0.05$); Compared with newborn C_{57} mice, the body of Mac-1$^{-/-}$ newborn mice showed obvious cyanosis. The specimens were taken from mice at 24 hours after birth (**Figure 1B**).

Alveolar layer thickening of Mac-1$^{-/-}$ mice

The lung tissue of C_{57} mice was floating in PBS buffer fluid, but for Mac-1$^{-/-}$ mice, some of the lung tissue was floating, most was falling down to the bottom of the tube due to increased density caused by insufficient lung expansion (**Figure 2A**). These phenomena indicated that Mac-1$^{-/-}$ mice may have respiratory failure at birth. And the results of lung tissue HE staining showed that the alveolar layer became thickening, monolayer alveolar cells became double and even multi-layer cells in Mac-1$^{-/-}$ mice. The lung tissue was drawn from the newborn mice at 24 hours after birth (**Figure 2B**).

The structure and function of type II alveolar cells in Mac-1$^{-/-}$ mice were relatively complete

To investigate the mechanism of the incidence of respiratory failure in newborn Mac-1$^{-/-}$ mice, expression of the specific protein proSP-C of type II alveolar epithelial cells was detected by immunofluorescence assay. **Figure 3A** and **3B** showed no significant difference of the expressions of proSP-C between C_{57} mice and in Mac-1$^{-/-}$ mice. Consistent with this, the results of Western blot showed that there was no significant difference in the expression of SP-C secreted protein between C_{57} and Mac-1$^{-/-}$ mice (**Figure 3C**). These results indicate that the structure and

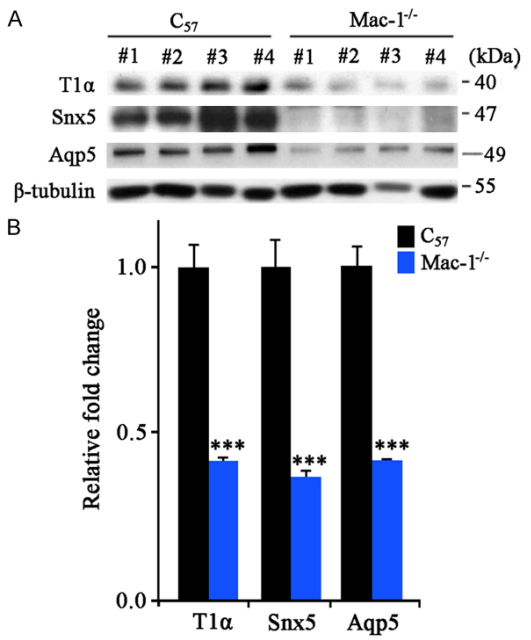

Figure 4. The specific proteins of type I alveolar cells, T1α, Aqp5 and Snx5 decreased significantly in Mac-$1^{-/-}$ mice. A. Showed the western blotting results, and B. Showed the statistical results. ***indicated P<0.001.

function of the alveolar surface of Mac-$1^{-/-}$ mice were not significantly different from that of C_{57} mice, and there was no significant difference between the expressions of alveolar surfactant proteins in these two kinds of mice.

The specific proteins of type I alveolar cells in Mac-$1^{-/-}$ mice decreased

The expression of specific proteins of type I alveolar cells, T1α, Aqp5 and Snx5, were detected. Western blotting results showed that compared with those in C_{57} mice, the expression of these three proteins in Mac-$1^{-/-}$ mice was decreased with statistical significance (P<0.001), indicating that the occurrence of respiratory failure may be related to the decrease of type I alveolar epithelial cells, as shown in **Figure 4**.

Discussion

Mac-1 is a member of β_2 integrin family, which, as an important adhesion molecule, is involved in body defense and immune response, as well as an important participant in inflammatory reaction, and inflammatory injury is an exact important factor in the lung injury [12]. It has been reported that Mac-1 plays an role in the occurrence of the lung disease [13], however, the study of Mac-1 in vivo experiment is very rare. This study investigated the effect of Mac-1 on respiratory failure. Compared with that of C_{57} mice, the perinatal mortality of Mac-$1^{-/-}$ mice was increased significantly, and the body of died mice showed obvious cyanosis, indicating that respiratory failure may be developed before mice died. In lung floating experiments, the lung tissue of Mac-$1^{-/-}$ mice was falling down to the bottom of the tube, indicating excessive lung tissue hyperplasia leading to density increase. It was not hard to find by pathological examination that the cause of mice death was very likely to be respiratory failure. Some studies have indicated that the respiratory failure is caused by the incomplete differentiation of type I or II alveolar epithelial cells, so we have a further study on alveolar epithelial cells.

To further explore the mechanism of lung injury, the specific markers of alveolar epithelial cells were detected, and results found that Mac-1 gene absence caused the impaired differentiation of type I alveolar epithelial cells, which resulted in the reduction of type I alveolar epithelial cells, eventually leading to the occurrence of respiratory failure. Other study has indicated that Mac-1 plays an important role in the maturation and differentiation of type I alveolar epithelial cells, so our study further investigated the role of Mac-1 in type I alveolar epithelial cells [14-17].

Type I alveolar epithelial cells cover more than 95% of the alveolar surface area, which are the target cells of various injuries. Besides participating the formation of air-blood barrier, they also transport water and ions, and have certain immune regulation functions [18, 19]. Type I alveolar cells are differentiated from type II alveolar cells, the mechanism of which in lung injury has been studied deeply [20]. Our study found that Mac-1 did not directly affect type II alveolar epithelial cells and the secretion of SP-C.

In conclusion, loss of Mac-1 affects the differentiation of type 1 alveolar cells and subsequent resulting in the incomplete of the lung structure and function, eventually leading to respiratory failure, suggesting the role of Mac-1 in the development and pathogenesis of respiratory failure.

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Disclosure of conflict of interest

None.

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